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Determination of the bioaccessible fraction of cupric oxide nanoparticles in soils using an *in vitro* human digestibility simulation.

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Abstract

This study investigated the bioaccessible fractions (BAFs) of Cu from copper-based nanomaterials present in soil to humans using an *in vitro* artificial simulation of the stomach; followed by the simulation of the small intestinal environment. The work compared the behaviour of coated and uncoated cupric oxide nanoparticles (CuO NPs) with $CuSO_4$ and the equivalent bulk CuO, and earthworms as a potential surrogate of human bioaccessibility. The calculated BAFs for the BGS 102 reference soil and the LUFA 2.2 soil (no added Cu) were ≤ 40%. In contrast, the LUFA 2.2 Cu-dosed soils measured statistically significant higher mean BAFs (ANOVA, $p < 0.05$); in general all above 60%. The calculated BAFs in the gastric phase did not differ statistically amongst the materials tested, both at low and high Cu dosing (ANOVA, $p > 0.05$). In the gastro-intestinal conditions, at the 200 mg Cu kg^{-1} soil concentration, the calculated BAFs for $CuSO_4$, bulk and nano CuO were 76.6%, 72.7% and 83.4%, respectively, and also did not differ statistically (ANOVA, $p > 0.05$). At the 1000 mg Cu kg^{-1} soil concentration, only the coated CuO NPs measured BAFs > 80%; with the COOH- and PEG-coated CuO NPs significantly more bioaccessible (ANOVA, $p < 0.05$) than all the other Cu-based materials. In terms of human health risks from ingested soil, this study did not show significant differences between soluble and particulate forms of Cu, but Cu concentrations from the gastro-intestinal phase digestion of soil were predicted by earthworm Cu concentrations.

Keywords: engineered nanomaterials, earthworms, copper, soil, BARGE

Introduction

Engineered nanomaterials (ENMs) acquire some of their novel properties from a typically high surface area to volume ratio, which influences both the physical and chemical behaviours of the materials.[1] Their unique properties at the nanoscale, including quantum chemistry and enhanced reactivity, are underling innovations in nanotechnology; with a continual increase in the manufacturing volumes of ENMs. Nanomaterials have found applications in new electronics, industrial coatings, textiles, building materials, medicines, cosmetics, food packaging and in chemical/biological remediation. In particular, copper-containing ENMs have been proposed as additives in animal feeds[2] and as components of antifungal biocides for agriculture use.[3]

Inevitably, ENMs will enter the environment and the predicted concentrations in surface waters are in the low $\mu g\ l^{-1}$ range or less, depending on the type of material.[4] However, an important final sink for ENMs is the soil environment.[5] ENMs may find their way into soils directly through the application of nano-enhanced biocides or fertilisers, from atmospheric deposition, leaching from streams, and also accidental releases. However, a main concern for the fate of ENMs is the application of sewage sludge to agricultural soils; where environmental concentrations of ENMs in sludge-amended soil are expected to be around the $\mu g\ kg^{-1}$ range.[6] Worse case predictions in the $mg\ kg^{-1}$ range have also been reported for soils.[7] Unfortunately, the quantification of ENM release into the environment, especially in complex matrices such as soil, is very challenging[8] and there is a dearth of field measurements from natural soils to confirm any predictions.

Soil quality is important to the health of terrestrial ecosystems, for agriculture, and for human health with respect to food safety and the incidental ingestion of soil. Consequently, there are guideline values for allowable total metal concentrations in soils. Some countries have set guideline values for total copper (Cu) in soils (e.g., Canada, 63 - 91 mg Cu kg^{-1}, CCME[9]; Finland, 100 – 200 mg Cu kg^{-1}, MEF[10]); but as yet there are no guideline values for nano forms of Cu, despite some predicted concentrations (e.g., $CuCO_3$ ENMs, 32 – 100 $\mu g\ kg^{-1}$, Gottschalk *et al.*[11]). For Cu and other metals, the hazard to wildlife and human health from ingested soil is not from the total metal content of the soil, but the bioavailable fraction that may be taken up internally by the organism. From an environmental chemistry perspective, the dissolved metal in the pore water and any labile metal easily removed from the soil grains might be regarded as

bioavailable. For ingested soil, the bioaccessible fraction is also considered. The precise distinction between 'bioavailable' and 'bioaccessible' fractions of contaminants is debated (e.g., Semple *et al.*[12]), but the bioaccessible fraction can be defined as the fraction released in the gut lumen during digestion that has the potential to be taken up by the organism.[13] In the context of human exposure to ingested soil, the bioaccessible fraction represents the maximum amount of contaminant that is available for intestinal absorption. Regardless of the definitions, these concepts were developed with the dissolved metal paradigm in mind, and as yet, it remains unclear if these notions can also be applied to ENMs.

It is estimated that children ingest 100 mg of soil a day[14], and this is a concern for human health risk assessments. Thus for the predictions of 100 μg kg^{-1} of Cu ENMs in soil above, this might represent a daily ingestion of 0.01 μg in the nano form. Copper is an essential nutrient, with humans requiring 1 - 2 mg of Cu day^{-1}, and under these normal circumstances the bioavailability of Cu salts is around 30 - 40% of the dose.[15] However, the gut is a protective barrier, and absorption declines exponentially with dose, so that only a few percent is bioavailable across the gut in potentially toxic situations.[15,16] Whether or not nano forms of Cu behave in this way is unclear, but for TiO_2 particles at least, the metal uptake rates across the vertebrate intestine are consistent with a bioavailable fraction of a few percent of the dose.[17]

Of course, it is not possible to conduct human oral exposure studies on contaminants, and for risk assessment purposes data on uptake of ENMs has been collected using oral gavage studies in rodents;[18] or *in vitro* models such as Caco-2 cells[19] and perfused intestines.[17] Animal studies should be limited in keeping with the ethical considerations of the 3Rs, but even the latter *in vitro* approaches require considerable technical expertise and these methods have not yet been standardised for regulatory toxicology. Alternatively, *in chemico* approaches that simulate the digestive processes in the lumen of the human gut have been available for many years and standardised with regulatory use in mind. The approach uses artificial saliva, gastric and intestinal juices to mimic the human digestive system from the oral cavity through to the small intestines; with adjustments of pH and additions of enzymes as appropriate for each region of the gut.[20] The large intestine is not simulated in these models, as it is assumed that most of the contaminant would be absorbed earlier in the digestive tract (an assumption not yet proven for nano). Nonetheless, this approach also known as '*in vitro* digestibility' by the nutrition discipline has been used to study metal releases from food,[21] and contaminated soils[22] so that the

bioaccessible fractions can be estimated. However, the simulated human digestion of soil has not been established for ENMs.

The aim of the present study was to determine the bioaccessible fraction of Cu from copper sulfate ($CuSO_4$) compared to pristine cupric oxide (CuO) ENMs and a bulk CuO powder in soil. In addition, the effect of surface coatings on the ENMs was investigated using a range of coatings on the common CuO core to represent anionic (carboxylate, COOH), cationic (ammonium, NH_4^+) and neutral ligands (polyethylene glycol, PEG). To add some environmental realism, a natural soil was used that had been subject to bioturbation by earthworms prior to the determination of the bioaccessible fractions in the soil. The unified bioaccessibility research group of Europe (BARGE) method[23] was selected for this work and adapted for ENMs. The method involved a two phase *in chemico* digestion process to simulate, (i) the mouth conditions and the low acidic environment of the human stomach, and (ii) the ensuing human upper intestinal with very mild acidic conditions.

Methodology

Soil preparation

The exposure of the soils was conducted as part of an earthworm acute toxicity test using an adaptation of OECD TG 207 for ENMs. The results of the earthworm ecotoxicity tests are reported elsewhere,[24] and the focus here is on the soil chemistry. Briefly, the experimental design included a control soil (no added Cu or ENMs), a metal salt control of Cu as $CuSO_4$ at 200 mg Cu kg^{-1} dry soil weight, the uncoated CuO ENM, and those coated with $–NH_4^+$, –COOH or –PEG respectively. The precise details of how the coatings were synthesised and attached to the ENM core is commercially sensitive information of the suppliers, but for clarity we use the term '$–NH_4^+$' to mean an $–NH_3$ terminal ligand that has been ionised with H^+ ions to achieve positive charge. The CuO ENMs were provided by PlasmaChem as part of the Nanosolutions EU project. The microscale (bulk) CuO material was obtained from BDH Chemicals Ltd, UK, and the metal salt ($CuSO_4.5H_2O$) from Sigma-Aldrich. The hypothesis was that the bioaccessibility potential of the copper dosed in the soil may be affected by the material type (e.g., nano *versus* bulk material or metal salt) and also coating-effects in case of the nanomaterials. For the ENMs, two test

concentrations were selected; a lower concentration of 200 mg Cu kg^{-1} soil representing around three times the expected background concentration of total Cu in European soils (the latter, ~ 60 mg Cu kg^{-1} , Heijerick *et al.*[25]). An upper concentration of 1000 mg Cu kg^{-1} equivalent to that suggested in the limit test for soil organisms according to OECD[26] was also used.

A standard sandy loam LUFA 2.2 (LUFA Speyer, Germany) soil was used with the following composition (supplier's information, mean ± SD, dry soil, *n* = not specified): pH of 5.5 ± 0.2 (measured in 0.01 M $CaCl_2$ solution); organic carbon, 1.8 ± 0.2%; nitrogen content at 0.17 ± 0.02%; and cation exchange capacity, 10.1 ± 0.2 meq 100 g^{-1}). The water-holding capacity of the soil was measured in-house and was 41.3 ± 3.0 g 100 g^{-1} dry weight. The soil used for the earthworm tests was sieved through a 2 mm mesh and air dried at 25 ºC. The soil pH was measured prior to the start and at the end of the experiment (in a 1:1 soil: water slurry, using a glass combination electrode, Corning 420), in addition to the metal composition (see below).

The ENMs, bulk CuO and $CuSO_4$ were mixed into the soil as dry powders by hand to ensure the test substance was evenly distributed, and the soils were then wetted to 50 - 55% water holding capacity (WHC) with ultrapure Milli-Q water (18.2 Ω). Four replicate boxes of soil *per* treatment were prepared and the soil was left to equilibrate with the moisture for one day prior to adding the earthworms. Adult *Eisenia fetida* (Savigny, 1826) with a typical mean starting wet weight of 5.5 ± 0.1 g (mean ± S.E.M, for a subsample of 12 of the initial earthworms) were exposed in 4 replicates (*n* = 12 earthworms *per* box, *n* = 48 earthworms *per* treatment) at 20 ± 1 ºC at 12:8 light:dark cycle.

After 14 days in the earthworm tests, approximately 30 g of wet soil was collected from each box and weighed (Sartorius BP 210) into previously acid washed (5% (*v/v*) nitric acid, Fisher, Primer Plus Trace Metals Analysis Grade) and deionised ceramic drying boats. The soil samples were then dried to constant mass at 85 ºC (Gallenkamp OV-160), allowed to cool to room temperature, and sieved to < 250 µm. The particle size fraction was chosen to represent the upper limit that is likely to stick to infants' hands.[27]

In addition to the natural soils from the earthworm tests, BGS Guidance Material 102,[28] which is an ironstone soil from Lincolnshire, England was also used to validate the analytical chemistry. This reference soil had not been used in the earthworm tests. In order to ensure complete soil re-homogenisation, the BGS 102 soil sample bottle was shaken manually for a few minutes before it was opened. The soil reference samples were digested and chemically analysed

strictly using the same approach applied to the LUFA 2.2 soil samples, but without any additions of Cu materials.

Characterisation of Nanomaterials

The characterisation included measurements of the primary particles sizes, the dispersion of the particles in ultrapure water and dialysis experiments to assess any dissolution of dissolved Cu from the particles. The CuO NPs were first examined using transmission electron microscopy (TEM, JEOL-1200EX II) for the primary particle size. Fresh stock suspensions, at 100 mg l^{-1} nominal concentration, were prepared in Milli-Q water and sub-samples were examined visually with $n = 60$ measurements of particle diameter *per* sample (conducted manually using ImageJ). The particle size distribution of the ENMs in the stock dispersions were also measured by nanoparticle tracking analysis (NTA) using a Nanosight LM 10 (Malvern Instruments, UK). Three sub-samples from each of the fresh stock suspensions were vortexed for 10 s immediately before analysis by NTA (Table 1).

Dialysis experiments were conducted in Milli-Q water at room temperature to measure the degree of copper metal ion dissolution from all the ENMs. Dialysis bags were filled with 8 ml of the appropriate test suspension at 100 mg l^{-1} nominal concentration, and suspended in a 600 ml beaker containing 492 ml of Milli-Q water (in triplicate beakers). Samples of 1 ml were taken from the external compartment of the beaker at time zero, 30 min, 1, 2, 3, 4, 6, 12 and 24 h for total Cu determination by ICP-OES or ICP-MS as appropriate. The data were subsequently fitted to a rectangular hyperbola (using SigmaPlot 13), and the maximum initial dissolution rate calculated from the maximum slope.

Aqua regia acid digestion of the soils, earthworms and nanomaterials

This was performed so that the total copper concentrations in the soil samples could be determined in order to facilitate calculations of the percentage of bioaccessible fractions. Briefly, a*qua regia* was prepared by adding 1 volume of > 68% concentrated nitric acid to 3 volumes of concentrated ~37% hydrochloric acid; both acids were of trace metal analysis grade (Fisher). This acidic mixture was allowed for a few minutes to develop into a golden coloured solution.

Then 10.0 ml of the *aqua regia* mixture was gently added into each 50 ml polypropylene tube containing 0.3 g of accurately weighed dried, sieved soil (n = 2 technical replicates, in accordance to INERIS[23]) from each box (n = 4 boxes) *per* treatment or control exposure. Two blank samples (without any soil) were analysed with every set of unknown samples. The tubes were heated with gentle mixing for 15 hours in a water bath set at 50 ºC. At the end of the heating time, each tube was mixed and its contents were allowed to cool down. Afterwards, 1 ml samples were taken from the clear upper part of each tube and diluted with 4 ml of 0.1 M nitric acid.

Acid digestion of the earthworms following day 7 and day 14 soil exposure is described elsewhere.[24] In addition, the original ENMs, bulk CuO and the equivalent metal salt, as dry powders, were also acid digested to verify their metal content. A known amount of powder (n = 3) was accurately weighed into a 20 ml polypropylene tube; three additional empty tubes with no material added to them were included as blanks. To each tube, 10.0 ml of the *aqua regia* mixture was gently added, followed by the same acid digestion method (see above) used for the soil samples.

Preparation of the synthetic gastro-intestinal digestive fluids

All the reagents used for the copper bioaccessibility determination in soil were of analytical grade, and are listed in Supplementary Table S1 for each type of synthetic fluid. The pH meter (Thermo Scientific Orion 2-Star Plus meter fitted with a Russell combination electrode) was precisely calibrated to pH 4.0, 7.0 and 10.0 at the start of each experiment. The synthetic digestive fluids (saliva, gastric, duodenal, bile) were prepared using sterile glass distilled water (distilled from ion-free ultrapure water, 18 MΩ resistance). The different fluid components (inorganic and organic, respectively) were placed separately on a magnetic stirring (IKA-WERKE R015) set at speed 3 for at least 3 h to ensure adequate mixing of each solution. Then, each digestive fluid was prepared by combining 250 ml of the inorganic and 250 ml of the organic components solutions, and with the addition of enzymatic components. Once the digestive fluid components were all mixed together, the fluids were allowed to acclimatise and stir for an hour at 37 ºC before use. Fig. S1 shows the serial additions of each type of fluid in relation to the steps in the digestion method.

236

237 ***Gastric phase and gastro-intestinal phase digestion***

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239 An outline of the adapted *in vitro* gastro-intestinal digestion protocol from INERIS[23] is depicted
240 in Fig. S1. As for the *aqua regia* acid digestion method (see above), 0.3 g of dried, sieved soil
241 was used for each bioaccessibility digestion (gastric phase and gastro-intestinal phase,
242 respectively). The same order of statistical replication (n = 2 technical soil replicate samples for
243 each soil box) was also followed for each digestion phase, including the use of two blanks.

244

245 ***Total copper determination***

246

247 The total copper concentration in the samples following *aqua regia* digestion or the adapted
248 BARGE methods were determined by inductively coupled plasma optical emission
249 spectrophotometry (ICP-OES, Thermo Scientific, iCAP 7000 Series), or equivalent mass
250 spectrophotometry (ICP-MS, Thermo Scientific, X Series 2). The instrument detection limit for
251 the ICP-OES was 0.008 mg l^{-1} Cu and for the ICP-MS was 0.003 mg l^{-1} Cu. Briefly, samples
252 were acidified, matrix-matched to the ICP-OES/ICP-MS standard metal solutions used for
253 calibration, with 0.8 mg l^{-1} yttrium as an internal standard. Sample blanks were included every
254 10 samples in each run of the instruments.

255

256 ***Calculation of the bioaccessible fractions***

257

258 The bioaccessible fraction (BAF) was calculated as a percentage of the total metal for each box
259 from the earthworm study, and for the BGS reference soil using Eq. (1);

260

261 $$BAF\ [\%] = \frac{\mathrm{Cu}_{\,bioaccessible}\ [\mathrm{mg\ Cu\ kg^{-1}\ soil}]}{\mathrm{Cu}_{\,total}\ [\mathrm{mg\ Cu\ kg^{-1}\ soil}]} \times 100 \quad (1)$$

262

263 where $\mathrm{Cu}_{bioaccessible}$ was the mean total copper concentration measured in the soil samples (n = 2
264 technical replicates within each soil sample from each box), following separately either the
265 gastric phase or the gastro-intestinal phase digestion, and Cu_{total} referred to the mean total

copper concentration measured from the *aqua regia* acid digestion (n = 2 technical replicates within each soil sample from each box).

Statistical analysis

The data are shown as mean ± standard error of the mean (S.E.M). The coefficient of variation (CV) was also calculated to describe the resultant percentage variability amongst the BAF values determined from the separate soil boxes (n = 4 boxes *per* treatment). All statistical analyses were carried out using IBM SPSS Statistics 22 and Microsoft Excel 2010. Following descriptive statistics, the Kolmogorov-Smirnov test was used to assess the normality of the distribution of data. Independent student *t*-tests and one-way analysis of variance (ANOVA, Tukey *post hoc* test) were used to check for significant differences amongst responses from within each test material and treatments. In instances where the data was not normally distributed, the non-parametric Mann-Whitney U test was used to assess differences between two independent groups. Likewise, the Kruskal-Wallis test was used as an alternative to a one-way between-groups analysis of variance. Figures were prepared using SigmaPlot 13.

Results

Particle characterisation and the total measured copper content in the test materials

The total measured Cu concentration in the different test materials as original powders is presented in Table 1, along with the details of purity, primary particle size and surface area of the materials investigated. The primary particle sizes of the test materials, as measured by transmission electron microscopy (TEM) images, were not found to exceed the manufacturer's reported size range (10 - 20 nm). Following dispersion of the test materials in water, the mean hydrodynamic diameter of the aggregates, as measured by nanoparticle tracking analysis were: 41 nm in the uncoated CuO NPs, 46 nm in the ammonium-coated CuO NPs, 121 nm in the COOH-coated CuO NPs and 100 nm in the PEG-coated CuO NPs. The dialysis experiments revealed some Cu dissolution from the different CuO NPs in ultrapure water. The dissolution rate of the uncoated CuO NPs was low (1.68 μg Cu h^{-1}, Table 1), but in comparison, all the

297 coated CuO NPs had higher dissolution rates; greater than 18 μg Cu h^{-1} (Table 1). However, the
298 rates were still micromolar, and even the highest rates would only equate to around 6 – 9% of the
299 total metal being released every hour.
300 On a mass basis of each material, the Cu content of material varied according to the
301 proportion of mass attributed to the coating. As a result of their chemical composition, less total
302 Cu was measured in the coated ENMs relative to the uncoated form (Table 1). For the coated
303 CuO NPs, the NH_4^+-coated NPs were found to contain the highest measured fraction of Cu
304 (0.52), followed by the COOH-coated NPs (0.43) and least Cu in the PEG-coated NPs (0.29).
305 Overall, the total Cu measurements in strong acid digests from the initial ENMs were reliable
306 with low coefficients of variations between replicates. Within-sample precision (triplicate
307 readings from the same sample) produced coefficient of variation values (CVs) ranging from
308 0.6% in $CuSO_4$ to 8.7% in the uncoated CuO NPs. The actual measured concentrations were
309 always a little less than the nominal concentrations. However, the calculated percentages, of the
310 actual measured concentrations relative to the nominal concentrations, for $CuSO_4$, bulk- and
311 nano-CuO were all above 85%.
312
313 ***Total measured copper concentrations in soil***
314
315 The exposure was confirmed by the measured total copper following the *aqua regia* digestion of
316 the soil samples (Fig. 1). The results of precision testing following the *aqua regia* acid digestion
317 in soil are presented in Supplementary Table S2, and the reproducibility of measurements
318 between boxes of soil was good. The unexposed control soils, without any addition of copper
319 (LUFA 2.2 soil and BGS 102 soil reference material), showed low Cu concentrations, as
320 expected (Fig. 1). All the Cu-dosed soils showed an increase in Cu concentration (Fig. 1) that
321 was consistent with the material types presented, and the relative proportions of Cu on a mass
322 basis expected in the different particle forms (Table 1). From correlation analysis (Fig. S2), a
323 positive relationship was also clear between the nominal and actual measured Cu concentrations
324 in soil following *aqua regia*, gastric and gastro-intestinal soil digestion. Furthermore, from all
325 soil extractions (Fig. 1), a statistical significant higher mean concentration of Cu in soil
326 (ANOVA, $p < 0.05$) was consistently measured in the *aqua regia* digests, as compared to the
327 gastric and the gastro-intestinal digests (milder digestion methods).

328 At the lower nominal 200 mg Cu kg^{-1} soil concentration (Fig. 1A), the measured
329 concentrations of Cu in the soil were not statistically different (ANOVA, $p > 0.05$) between the
330 gastric and the gastro-intestinal digests; with the exception of the uncoated CuO NPs dosed soils
331 that measured a higher mean concentration of Cu following the gastro-intestinal phase digestion.
332 At the 1000 mg Cu kg^{-1} soil concentration (Fig. 1B), the measured mean Cu concentrations from
333 bulk CuO and the uncoated CuO NPs in the soil digests, following the gastric phase and the
334 gastro-intestinal phase digestion did not differ (ANOVA, $p > 0.05$). In contrast, all coated CuO
335 NPs digests were found to have higher levels of Cu following the gastro-intestinal phase
336 digestion, in comparison with the gastric phase digestion.
337
338 ***Calculated bioaccessible fractions***
339
340 A relatively high Cu concentration measurement in soil was not found to necessarily represent a
341 greater bioaccessibility potential for that metal in soil, as evident from the gastro-intestinal
342 digestions (Fig. 2). At both the 200 and 1000 mg Cu kg^{-1} soil concentration, higher overall BAF
343 values were calculated from the gastro-intestinal phase relative to the gastric phase digestion
344 (Fig. 2, Table S3). However, in terms of the individual Cu-materials tested (Fig. 2) there were a
345 few statistical differences between the calculated gastric and gastro-intestinal BAFs (*t*-test, $p <$
346 0.05). Mean BAFs greater than 80% were only recorded in the gastro-intestinal phase digestion
347 (Fig. 2, Table S3) for the uncoated CuO NPs (low dose exposure) and in the COOH-, PEG- and
348 NH_4^+-coated CuO NPs (high dose exposure).
349
350 ***Gastric phase digestion BAFs***
351
352 Following the gastric phase digestion, the calculated mean percentage BAFs for the control BGS
353 102 soil and the LUFA 2.2 soil (with no added copper) were 35.3% and 38.8% respectively
354 (Table S3, Fig. 2). These two mean BAF values were not significantly different (ANOVA, $p >$
355 0.05); whereas all LUFA 2.2 soils dosed with Cu were found to have much higher percentage
356 BAF values, ranging from a minimum of 53.5%, a maximum of 98.1% and a median value of
357 69.3%. Irrespective of the initial nominal soil input Cu concentration (low or high), all soil

treatments with CuO NPs, or with bulk CuO or copper sulfate in the gastric phase digestion (Fig. 2) did not differ significantly in their calculated percentage BAF (ANOVA, $p > 0.05$).

Gastro-intestinal phase digestion BAFs

The calculated Cu BAF values in soil following the gastro-intestinal phase digestion are in general comparable to the outcome following the gastric phase digestion (Figs. 2A and B). However, a greater distribution of the percentage BAF values was evident from the gastro-intestinal phase digestion. Percentage BAFs ranged from a minimum of 38.7%, a maximum of 96.7% and a median of 73.4%. The mean Cu percentage BAFs determined for bulk CuO and the uncoated CuO NPs in the gastro-intestinal phase digestion, did not differ statistically between the low and high Cu dosage (ANOVA, $p > 0.05$); but higher mean BAF values were calculated at the 200 mg Cu kg^{-1} soil concentration (Figure 2A). The opposite was true for all coated CuO NPs, where higher mean percentage BAFs resulted at the 1000 mg Cu kg^{-1} soil concentration (Fig. 2B).

Calculated BAFs relative to metal dissolution and uptake in earthworms

In Fig. 3, the concentration of total Cu in the soil (Fig. 3A) and the percentage of bioaccessible fraction (Fig. 3B) for the gastro-intestinal phase were plotted against the maximum dissolution rates of copper following dialysis experiments of the different test materials in Milli-Q water. Dissolution rate was inversely related to gastro-intestinal phase Cu concentration, with the trend most evident at the soil dose of 1000 mg Cu kg^{-1} soil (Fig. 3A). However, there was no clear relationship between the percent of BAF and metal dissolution at the 200 mg Cu kg^{-1} soil concentration (Fig. 3B). At the higher soil exposure dose, an increase in metal dissolution rate coincided with higher measurements of gastro-intestinal BAFs, in the order of: uncoated CuO NPs, NH_4^+-coated CuO NPs, PEG-coated CuO NPs and COOH-coated CuO NPs (highest).

In contrast to the dissolution rate data, there was a clear correlation between the gastro-intestinal phase Cu concentration and the Cu concentration in the earthworms (Fig. 3C), with an r^2 value of 0.94 for all the data, regardless of exposure concentration. However, this relationship was lost when the data were presented as the calculated gastro-intestinal BAFs and Cu

concentrations in the earthworms following 14 days of exposure (Fig. 3D). At the 200 mg Cu kg^{-1} soil concentration, no clear pattern was evident between the calculated BAFs and earthworm copper concentration. However, at the higher exposure dose in the soil (1000 mg Cu kg^{-1} soil concentration). The measured BAF values for the COOH-, NH_4^+-coated CuO NPs and the uncoated CuO NPs were however inversely proportional to the measured Cu content in earthworms. The order of measured metal concentration in the earthworms by coating also coincided with the relative amount of copper in the different test materials (see Table 1).

Discussion

This study reports the BAF of Cu from cupric oxide nanoparticles with different surface coatings, compared to the metal salt and bulk powder controls, using the *in vitro* human gastro-intestinal BARGE method. Overall, the data shows that there is a bioaccessible fraction of Cu (form unknown) from all the materials tested, and this broadly follows the notion of dose-response with more total metal available at the higher nominal concentrations in the soil. Crucially, there was a material-type effect that was also dependent on the phase of digestion. In the gastric phase, the BAF were similar at around 70% of the total metal, regardless of the material tested. However, in the gastro-intestinal phase some material-type effects were revealed; with the CuO NPs sometimes having higher BAFs than the metal salt. While the BAF values correlated well with the original total measured Cu concentrations in the soil, they were not easily explained by the dissolved metal paradigm. For the nanomaterials, there was no correlation between the BAF and the dissolution rate of the particles. Moreover, the bioaccumulation pattern in earthworms from the same soils did not correlate easily with BAF values either. Only when the absolute Cu concentration from the gastro-intestinal phase was plotted against the Cu concentration in the earthworms was a correlation revealed; indicating that metal concentration in earthworms might be a possible surrogate of the human health risks from ingested soil for these nanomaterial.

Validation of the unified BARGE method for ENMs

The unified BARGE method is a relatively well-standardised and validated method for determining the bioaccessible fractions of metals in soils. The BARGE method was originally devised with the concern for metal exposure associated with incidental soil ingestion in humans, especially children, in mind.[22] It has since been used to test a variety of soils for bioaccessible metals.[29-31] However, it has not been specifically validated for ENMs. The current investigation attempted to validate the BARGE method using several approaches including: (i) measuring the BAF for Cu metal in a BGS 102 soil reference; (ii) determining the BAF for $CuSO_4$ in a well-known LUFA 2.2 soil, and then, (iii) exploring the within sample and between sample reproducibility of the BARGE method for $CuSO_4$ compared to the ENMs.

The performance of the BGS 102 soil reference material was considered first. This soil already contains some naturally occurring Cu, and so no additional Cu amendments were necessary. The measured total Cu in this soil was 17 mg kg^{-1} in comparison to 26 mg kg^{-1} from Wragg[28] (see Table S2). In addition, the BAF values were 35% in the gastric phase and 40% in the gastro-intestinal phase for Cu (Table S3). These BAF values are entirely consistent with previous findings from Hamilton *et al.*[32], with a mean reported Cu BAF of 33%. Furthermore, measurements of the BGS 102 reference soil were within acceptable limits for a standard method, with coefficients of variation being 10 % or much less (Table S3).

The LUFA 2.2 soil is also relatively well-known and has been widely used in soil ecotoxicity testing with earthworms. Its natural mean Cu content is low (3 mg Cu kg^{-1} soil, Table S2), in agreement with measured metal concentrations in uncontaminated soils.[33] Criel *et al.*[34] reported a background total Cu concentration in the LUFA 2.2 soil of 6 mg Cu kg^{-1}. In the present work, the measured BAFs in the LUFA 2.2 soil (32 - 39%) were comparable with the calculated BAFs of the soil reference BGS 102 soil (Fig. 2), and other studies from natural uncontaminated soils.[29]

Engineered nanomaterials do not behave in the same way as solutes,[35] and as 'difficult to handle' substances, they present a number of challenges to the standard methods used in regulatory testing (reviews, Handy *et al.*[36]; Selck *et al.*[37]). One concern is whether or not the sum of the difficulties in maintaining the exposure, detailing the heterogeneous nature of the materials in biologically-relevant matrices such as soil, and any losses during the analytical procedures for detecting ENMs, etc., cause such high variation between replicates to the extent that the overall attempt at standardisation fails. Of course, the notion of acceptable deviation in a standardised

method depends on the context. In this study, the LUFA 2.2 soil was amended with additions of $CuSO_4$, bulk CuO and CuO NPs, respectively. The behaviour of Cu^{2+} ions in soil is relatively well-known and the analytical methods for measuring total Cu in soil samples is established. The between sample deviation reflected this with CVs ranging between 8 - 13% for the calculated BAF values for $CuSO_4$ (Table S3). Furthermore, despite the challenges of handling ENMs, the CVs for the particulate forms of Cu were in the same range (Table S3). The only exceptions were the CuO-PEG NPs which showed variations as high as 26%, and the bulk CuO material with CVs ranging between 7 - 26%. While these latter variations are not as low as one would prefer (ideally, <10 %), they are not beyond acceptability from the view point of standardised protocols for environmental testing. For example, the Organisation for Economic and Cooperation and Development (OECD), allows a 20% deviation in the measured test concentrations in valid acute ecotoxicity tests,[36] even greater deviations in test methods are allowed for 'difficult to handle' substances that are not miscible with water.[38]

The bioaccessibility of copper sulfate in soil

There are many studies that use the BARGE method or similar approaches to measure extractable Cu from metal-contaminated soils (review, De Miguel *et al*.[30]). However, to our knowledge, only the present study has specifically assessed bioaccessible fractions of Cu from $CuSO_4$ dosing to the LUFA 2.2 soil using the BARGE method. Soil dosing with $CuSO_4$ at the 1000 mg Cu kg^{-1} soil concentration was not undertaken here, as the metal salt at such high doses is known to be toxic to invertebrates, including earthworms.[39] At the 200 mg Cu kg^{-1} soil concentration, as anticipated, the measured total Cu from $CuSO_4$ was close (99%) to the nominal concentration (Table S2). The calculated mean BAF for the metal salt (Table S3, Fig. 2) did not differ (*t*-test, $p > 0.05$) between the gastric (73%) and the gastro-intestinal phase digestion (77%). These values are much higher than found by simpler $CaCl_2$-extractable Cu measurements in contaminated LUFA 2.2 soil where only about 30% or less of the Cu is labile.[40] The fact that the Cu from $CuSO_4$ was predicted as bioaccessible to both the stomach and the intestines is not surprising given the solubility of the metal salt. However, the uptake of dissolved Cu by the gut also depends on the anatomical locations of the necessary Cu transporters in the gut epithelium. Pharmacological studies with gut preparations of vertebrate animals show it is the intestines, not

482 the stomach involved in Cu uptake, and that the uptake mechanisms include a luminal chloride-
483 dependent pathway.[16] Similarly, *in vivo* studies with rodents using radiolabelled Cu show the
484 intestine as the main location for dietary Cu uptake.[41] Thus for Cu, the BAF does not necessarily
485 indicate a hazard, only that the metal may become hazardous if the BAF is present in the
486 intestines where the Cu transporters occur.
487
488 ***Are particulate forms of Cu more bioaccessible than $CuSO_4$?***
489
490 There have been some studies on the dissolution of ENMs in the presence of gastro-intestinal
491 fluids. For example, with Ag NPs,[42,43] silica NPs[44] and CdSe QDs.[45] However, these studies were
492 more focused on the physico-chemical properties and aggregation behaviours of the ENMs in the
493 digestive juices. In the gastric phase, the calculated BAFs from all the test materials, including
494 the metal salt, were not statistically different and remained around 70% (Fig. 2). There was no
495 evidence of any difference between the particles and the metal salt that might infer a particle
496 size-effect, and no particle-coating effects (Fig. 2, Table S3). Arguably, this observation for the
497 stomach could be explained by the strong acid (pH < 1.5) simply dissolved the different
498 materials at similar rates; regardless of their surface areas or aggregate sizes (Fig. 1). The rapid
499 dissolution of Cu NPs at low pH has also been observed in studies with freshwater fish,[46] in acid-
500 extractions of soil during earthworm studies,[24] and at low pH in the physiological salines used
501 for oral gavage in rodents.[47] Thus, *in vivo* the Cu NP are likely transformed into soluble Cu in the
502 stomach, which is then absorbed in the intestine, and then can accumulate in the internal organs
503 and have toxic effects, albeit with some slight delay compared to oral gavage with the metal
504 salt.[47]
505 However, the Cu bioaccessibility from soil was generally was similar for each substance
506 in both the acidic gastric phase and the neutral gastro-intestinal phase (Fig. 2, Table S3). There
507 was no metal salt and or nanomaterial effects, apart from the CuO-PEG material which showed
508 less bioaccessibility in the gastro-intestinal phase at the 200 mg Cu kg^{-1} soil concentration (Fig.
509 2A). The reduction was only a few % change, and one might argue that this is of limited
510 biological importance. However, the mechanism behind this effect for a PEG-coated material is
511 unclear. It might be that the PEG is more stable at neutral pH and vulnerable to some acid
512 degradation in the gastric phase. Regardless, this reduction in BAF is also consistent with the

same CuO-PEG material causing only moderate Cu accumulation in earthworms ingesting contaminated soil compared to the other coatings after 14 days, and no appreciable mortality.[24]

At the 1000 mg Cu kg^{-1} soil concentration, the BAF values were greater for the coated CuO NPs in the gastro-intestinal phase relative to the gastric phase; and compared to the uncoated CuO and the bulk material (Fig. 2B). This apparent effect of the coated materials to be more accessible in the gatro-intestinal phase needs more investigation, but might be explained by the nanoparticle coatings absorbing (electrostatic attraction in the case of –COOH) or becoming associated with (e.g., by steric hindrance in the case of –PEG) macromolecules present in the gut digestive juices such as proteins. This might, in theory, render their surfaces more bioaccessible than that of the uncoated NPs, through the rapid initiation of a corona.[48] *In vivo*, the CuO-COOH was the most toxic to earthworms in fresh soil,[24] in keeping with it also being one of the more bioaccessible forms here in the soil. Unfortunately, as yet, there are no *in vivo* studies with mammals to confirm if the coating-effects for CuO NPs observed in earthworms might also apply to humans.

Conclusions and implications for human health risk assessment

Human health risk assessment from soils considers the total and the bioaccessible fraction. The BARGE approach used here gave the expected findings for reference soils and those spiked with $CuSO_4$. The methodology also performed well for CuO-based NPs. This study has shown that the bioaccessible fractions of Cu are similar for $CuSO_4$, the CuO bulk material and most of the forms of the CuO NPs (Fig. 2); suggesting that the existing human health risk assessment for the ingestion of Cu in soil may also be protective of particulate forms. For the coated CuO NPs, the bioaccessible fraction was greatest at the high exposure concentration; implying that the BAF is not fixed for nanomaterials, but dependent on dosimetry. The greatest hazard was arguably presented in the gastro-intestinal phase at the highest concentrations of the different CuO NPs used, where BAF values were around 70% or more (Fig. 2B). The BARGE method here is a simulated digestion in the human gut without food, and therefore represents a worst case scenario for potential uptake. It is also unfortunate that the greater bioaccessibility in the gastro-intestinal phase is also coincident with the intestinal location of copper transporters involved in absorption across the gut.[49] However, it is clear that the dissolved fraction of the metal from the

particles did not correlate easily with the BAF values in the present study. This strongly suggests that the bioaccessible fraction includes a particulate load. Further work is needed to confirm this and the gastrointestinal locations of any CuO NP uptake *in vivo*. Finally, the BARGE method here is intended for human health risk assessment, and this might therefore be followed by *in vivo* dietary studies on mammals when a concern for bioaccessibility has been identified. From an animal welfare perspective, dietary toxicity tests on vertebrate animals are to be avoided where possible. The alternative approach of using surrogate soil organisms such as earthworms to predict the dietary bioaccessibility of metal in soil to humans has some merit. Button *et al*. [50] found that BAF values for arsenic in soils correlated with total As accumulation in earthworms. Similarly in the present study, the measured Cu remaining in the soil following the gastro-intestinal phase correlated with the Cu concentrations in the worms (Fig. 3C). However, care must be taken with the choice of data as the Cu accumulation in the earthworms was not predictive of the calculated gastro-intestinal BAF when expressed as percentages (Fig. 3B). Only the measured concentrations should therefore be used in any read across attempt from earthworms to humans for the human health risk assessment for soils.

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Declaration of interest

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723 **Table 1** Test materials characterisation from the original powders.

Test material (Supplier)	Manufacturer's information	[1]Measured primary particle size (nm)	[2]Measured hydrodynamic diameter in ultrapure Milli-Q water (nm)	[3]Total measured copper concentration (mg l^{-1})	Between-replicate percentage CV (%)	[4]Percentage of nominal concentration (%)	[5]Measured copper fraction in coated CuO NPs	[6]Metal dissolution rate in water (µg Cu h^{-1})
$CuSO_4.5H_2O$, CAS 7758-99-8 (Sigma-Aldrich 31293 , Lot SZBC0170V)	Purity, 99 - 102%	--	--	102.7 ± 0.4	0.6	100.8 ± 0.4	--	---
CuO Bulk, CAS 1317-38-0 (British Drug Houses Ltd)	Analar grade	---	---	285.0 ± 13.4	8.1	89.1 ± 4.2	--	---
[#]CuO NPs uncoated, CAS 1317-38-0 (PlasmaChem GmbH, Lot YF1309121)	99% purity; diameter, 10 - 20 nm; [$]surface area 42 ± 2 m^2 g^{-1}	12.00 ± 0.37	41 ± 28	287.1 ± 14.4	8.7	89.7 ± 4.5	--	1.68
[#]CuO NPs COOH-coated, CAS 1317-38-0 (PlasmaChem GmbH, Lot YF140114)	99% purity; diameter, 10 - 20 nm; [$]surface area, 7.4 ± 0.5 m^2 g^{-1}	6.45 ± 0.16	121 ± 91	154.3 ± 6.9	7.7	-	0.43 ± 0.02	69.12
[#]CuO NPs NH_4^+-coated, CAS 1317-38-0 (PlasmaChem GmbH, Lot YF140114)	99% purity; diameter, 10 - 20 nm; [$]surface area, 6.1 ± 0.5 m^2 g^{-1}	9.53 ± 0.22	46 ± 36	185.9 ± 7.2	6.7	-	0.52 ± 0.02	18.6
[#]CuO NPs PEG-coated, CAS 1317-38-0 (PlasmaChem GmbH, Lot YF140114)	99% purity; diameter, 10 - 20 nm	7.46 ± 0.42	100 ± 36	105.0 ± 3.5	5.8	-	0.29 ± 0.01	52.02

[#]Supplied as dry powders, bespoke design and production of spherical particles for the NANOSOLUTIONS project *via* Alexei Antipov, PlasmaChem GmbH; [$]Brunauer–Emmett–Teller (BET) surface area values (mean ± one standard deviation, $n = 3$) from NANOSOLUTIONS project; [1]Based on transmission electron microscopy (TEM) images of CuO ENMs from a 100 mg l^{-1} Cu stocks in Milli-Q water where data are mean ± standard error of the mean (S.E.M) with $n = 60$ measurements; [2]Particle size distribution measurements (mean ± one standard deviation, $n = 3$) by Nanoparticle tracking analysis (NTA) on 100 mg l^{-1} Cu ENM stocks in Milli-Q water; [3]Data are means ± S.E.M ($n = 3$ replicates) of total measured copper concentration by ICP-OES following *aqua regia* acid digestion of the dry powders, and after normalisation to an initial 0.02 g weight of material; Cupric oxide nanoparticles (CuO NPs); Coefficient of variation (CV); [4]With a 0.25 fraction of copper by weight in $CuSO_4.5H_2O$, and 0.80 fraction of copper in both CuO bulk and uncoated CuO NPs; [5]Relative to the measured copper content in the uncoated CuO NPs; [6]Maximum slope from rectangular hyperbola function of curve fitting used to estimate the maximum rate of dissolution of copper from the dialysis experiments, in triplicate;.- Not possible to calculate from the manufacturer's information on material composition; -- Data not applicable to the test material; --- Not measured.

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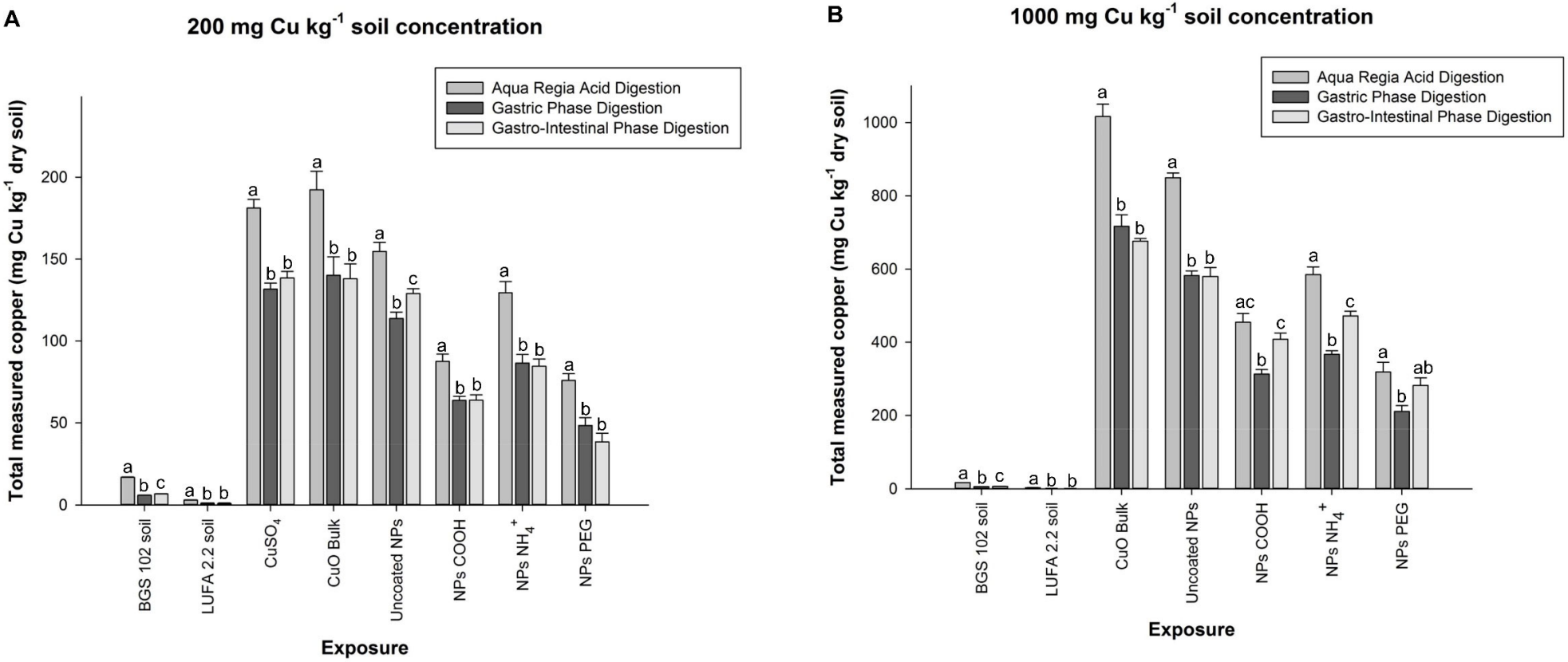
Figure Legends

Fig. 1 Total measured copper concentration (mg Cu kg^{-1}) in dry soil, from the different treatment exposures at day 14 in the earthworm tests, following *aqua regia* acid digestion, gastric phase and the gastro-intestinal phase digestion, respectively; (A) at the 200 mg Cu kg^{-1} soil initial nominal dosing and (B) at the 1000 mg Cu kg^{-1} soil initial nominal dosing. Materials labelled as BGS102 soil and LUFA 2.2 soil, refer to control soils (no added Cu or ENMs). The BGS 102 soil was not used in the earthworm tests, but solely included to validate the analytical chemistry. Data are mean ± S.E.M (n = 8). Different letters indicate significant differences within each material (ANOVA, $p < 0.05$). Observed differences in soil measured Cu amongst the test materials, with varied initial relative mass proportion of Cu, are not identified with statistical labels.

Fig. 2 Calculated percentage bioaccessible fraction (BAF) from the different treatment exposures at day 14 in the earthworm tests, following the gastric phase and the gastro-intestinal phase digestion, respectively; (A) at the 200 mg Cu kg^{-1} soil initial nominal dosing and (B) at the 1000 mg Cu kg^{-1} soil initial nominal dosing. Materials labelled as BGS102 soil and LUFA 2.2 soil, refer to control soils (no added Cu or ENMs). BGS 102 soil was not used in the earthworm tests, but solely included to validate the analytical chemistry. Data are mean ± S.E.M from (n = 4) separate soil boxes *per* treatment. Different letters in panel (A) or (B) indicate significant differences amongst the relative tested materials (ANOVA, $p < 0.05$) in gastric or gastro-intestinal phases, respectively. *, in panel (A) or (B) refers to a statistical significant difference in calculated BAF between gastric and gastro-intestinal phases in that relative test material and concentration (t-test, $p < 0.05$).

Fig. 3 The relationship between the mean total measured copper concentration in the soil following the gastro-intestinal digestion (left-hand panels), or the mean percentage of the gastro-intestinal bioaccessible fractions (BAFs) of Cu (right-hand panels), plotted against the measured copper dissolution rates of the ENMs in Milli-Q water (panels A and B), or the total mean copper concentration in the earthworms at day 14 (panels C and D). Data on the *y-axis* are from n = 4 separate soil boxes, at either 200 or 1000 mg Cu kg^{-1} soil concentration, and n = 8 earthworms *per* treatment, except for NPs COOH at high dose where n = 2 as a result of high animal mortality (*x-axis*).

Fig. 1


A
200 mg Cu kg-1 soil concentration
Aqua Regia Acid Digestion
Gastric Phase Digestion
Gastro-Intestinal Phase Digestion
Total measured copper (mg Cu kg-1 dry soil)
0
50
100
150
200
a
b c
a b b
a
b b
a
b b
a
b b
a
b b
a
b b
a
b b
BGS 102 soil
LUFA 2.2 soil
CuSO4
CuO Bulk
Uncoated NPs
NPs COOH
NPs NH4+
NPs PEG
Exposure
B
1000 mg Cu kg-1 soil concentration
Aqua Regia Acid Digestion
Gastric Phase Digestion
Gastro-Intestinal Phase Digestion
Total measured copper (mg Cu kg-1 dry soil)
0
200
400
600
800
1000
a
b c
a b b
a
b b
a
b b
ac
b c
a
b c
a
b ab
BGS 102 soil
LUFA 2.2 soil
CuO Bulk
Uncoated NPs
NPs COOH
NPs NH4+
NPs PEG
Exposure

759 **Fig. 2**

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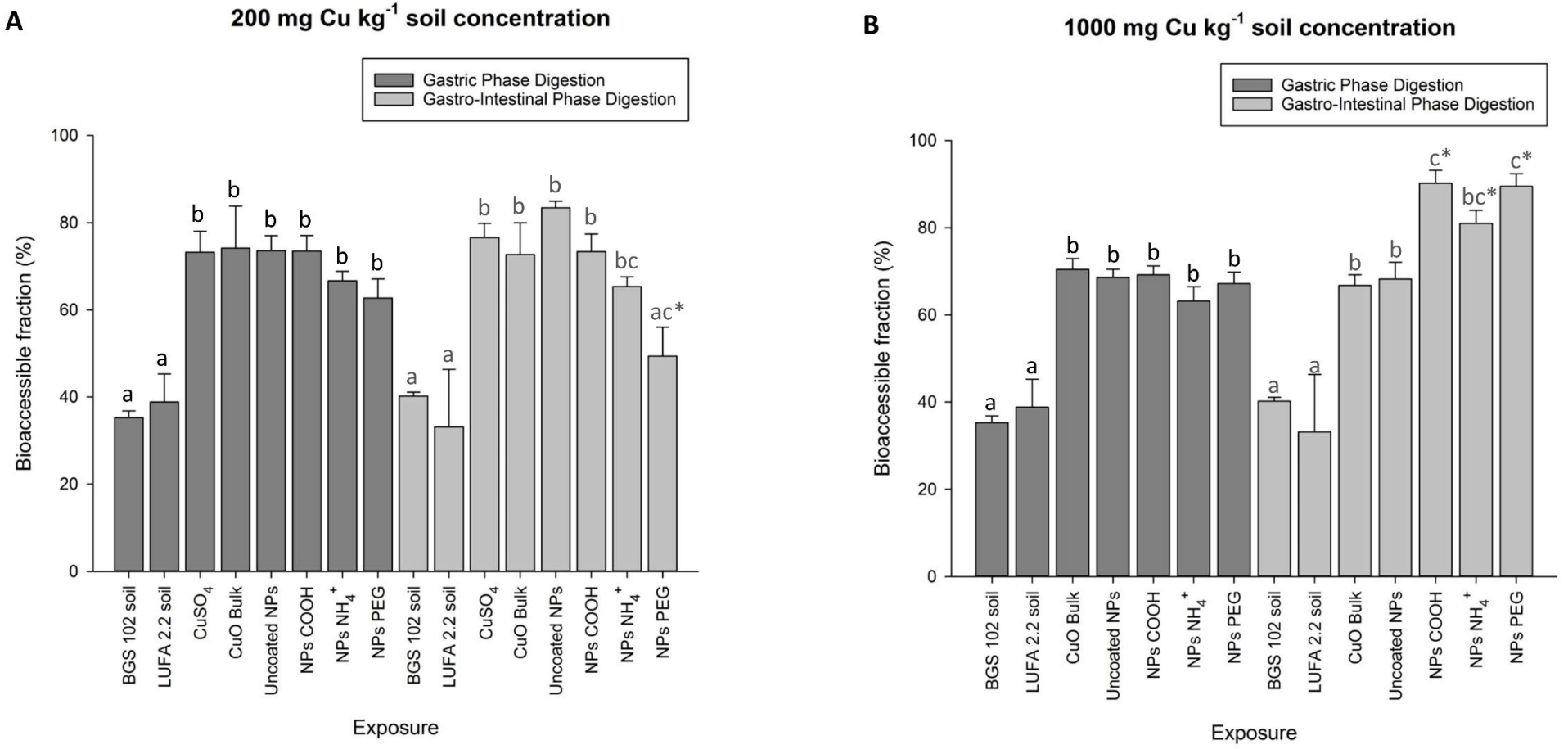

761

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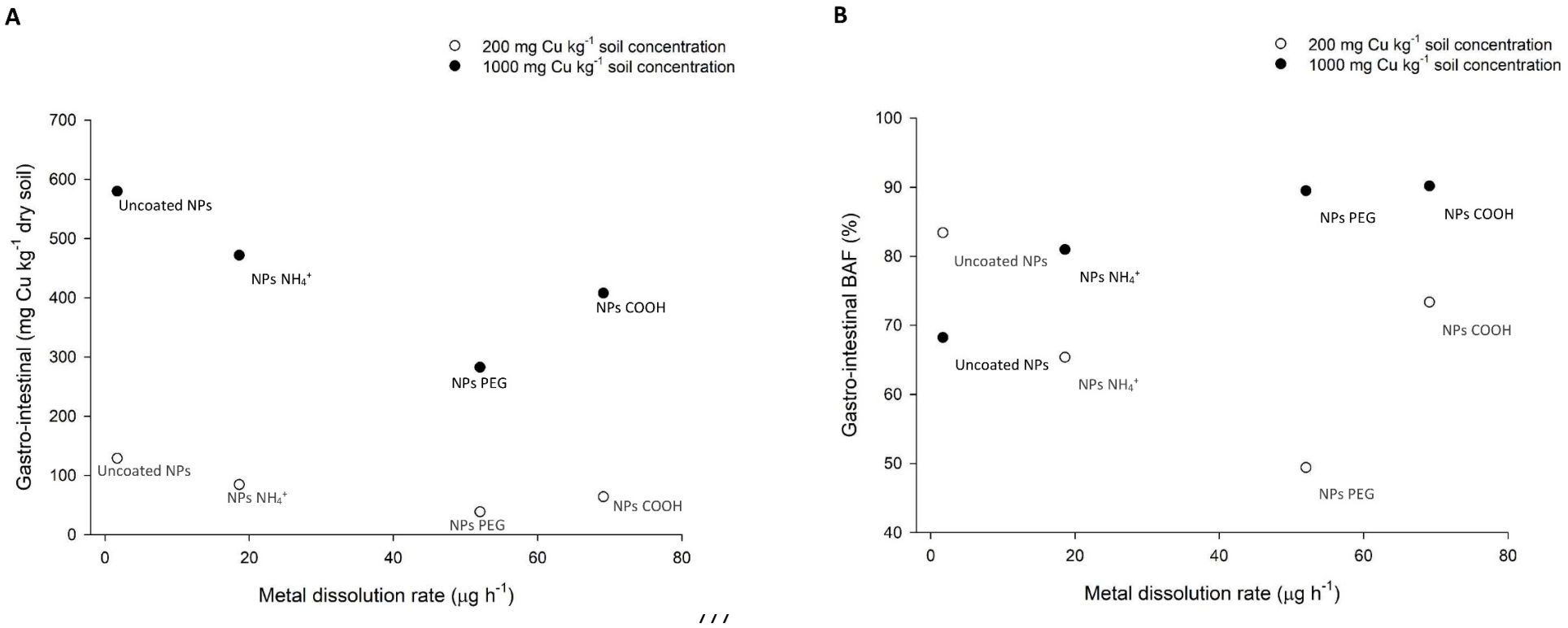
763

764 **Fig. 3**

765



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782 **Fig. 3 cont.**

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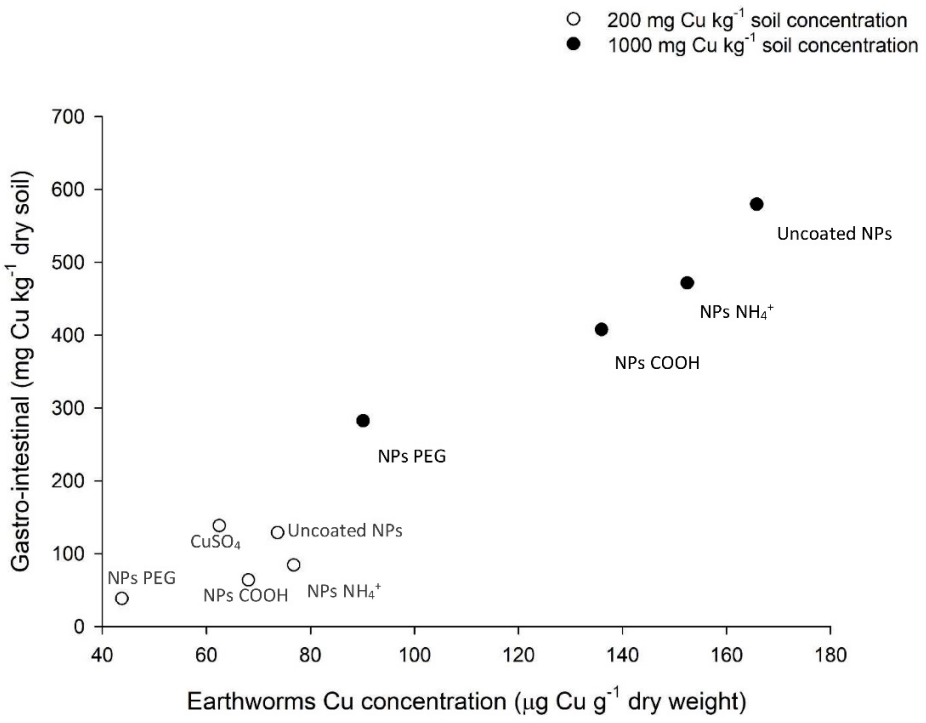
C



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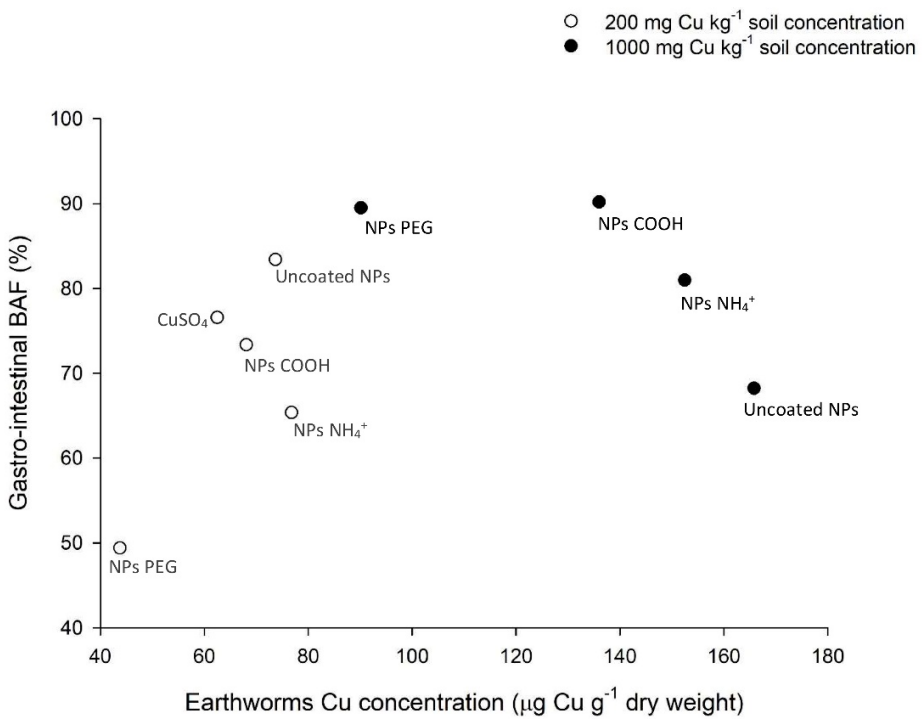

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